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**Article type:** Review

## **Advances in Affinity Ligand-Functionalized Nanomaterials for Biomagnetic Separation**

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## **Abstract**

The downstream processing of proteins remains the most significant cost in protein production, and is largely attributed to rigorous chromatographic purification protocols, where the stringency of purity for biopharmaceutical products sometimes exceeds 99%. With an ever burgeoning biotechnology market, there is a constant demand for alternative purification methodologies, to ameliorate the dependence on chromatography, while still adhering to regulatory concerns over product purity and safety. In this article, we present an up-to-date view of bioseparation, with emphasis on magnetic separation and its potential application in the field. Additionally, we discuss the economic and performance benefits of synthetic ligands, in the form of peptides and miniaturized antibody fragments, compared to full-length antibodies. We propose that adoption of synthetic affinity ligands coupled with magnetic adsorbents, will play an important role in enabling sustainable bioprocessing in the future.

Keywords: superparamagnetic, micro-particles, nanoparticles, bioseparation, peptides, antibody fragments

## *1. Introduction*

Protein downstream processing can be divided into four main stages, based on the objective to be achieved at each stage. These four stages describe recovery, concentration, purification and formulation (Nfor et al. 2008). The third stage in the process, purification, currently represents the bottleneck in the biopharmaceutical production of proteins, and represents anywhere from 30-80% of the production costs. Commonly, to reach the desired level of purification, multiple chromatographic steps are required, with the stringency of purity required for pharmaceutical and therapeutic proteins sometimes exceeding 99 % (Headon and Walsh 1994). Chromatography currently represents the favorite choice as a result of the high resolution it offers. Nevertheless, there is an on-going search for new and improved alternatives to chromatography in an effort to lower costs and improve yield, while maintaining high product purity.

Improved cell lines, culturing media and growth conditions have allowed upstream processing to continue to thrive. Downstream processing, by comparison, struggles to keep up. Mammalian expression levels of 1 g/L are now commonplace (Langer 2007a), and expression levels as high as 27 g/L have been reported (DSM and Crucell 2008). As a result, 47% of European biomanufacturers report bottleneck issues associated with downstream processing (Langer 2007b). While efforts to reduce cost and improve affinity performance by utilizing synthetic affinity ligands can assist, the current bottleneck in downstream production will ultimately be alleviated by the adoption of novel separation technologies. Despite high cost, difficulty in scaling-up and low throughput, bioseparations are still dominated by chromatographic approaches. For therapeutic application, the high resolution afforded by packed-bed chromatography is unrivalled. However, industrial large scale chromatographic purification of protein is not without its issues. Capture of 100 kg of an antibody, assuming a loading capacity of 50 g/L, requires a 2,000 L column, with a diameter of 3.2 m and a 25 cm bed height. In industrial settings today, larger columns can be up to 2m in diameter with bed heights of 10-20 cm (Thömmes and Etzel 2007). The weight of the adsorbent material in large columns can negatively impact on the nature of the adsorbent, causing it to become distorted and squashed. In smaller columns, the interaction of the adsorbent beads with the wall of the column will have a supportive effect over a short distance, but wider columns lose this supportive effect in the middle, distorting the stationary phase and restricting flow. An alternative is to perform the purification in multiple cycles to reduce the amount of required stationary phase. With this approach, one must consider the finite amount of useful cycles a stationary phase may offer. Furthermore, on the large scale, packed-bed chromatographic

separations require large volumes of liquids for equilibration, washing, elution, regeneration and sanitization, e.g., for a 2,000 L affinity column, more than 50,000 L of buffers may be required (Thömmes and Etzel 2007). Elution of protein at such a large scale poses additional problems. Protein eluates in low pH buffers have a tendency for aggregation, causing loss of functionality and generating immunogenic aggregates. Removal of these unwanted side-effects adds an extra purification step, thereby increasing cost and decreasing yield. The chromatographic process is further let down by slow adsorption and separation of the target molecules in the stationary phase. Variation in column length and sample volume mean adsorption and separation can take anywhere from hours to a day, or even more. Additionally, packed-bed chromatographic columns cannot tolerate particulate matter which would block the column. Consequently, chromatography is not suitable for the purification of samples from early processing stages, where the presence of minute suspended solids is commonly encountered. To address these issues, expanded-bed chromatography (EBA) has been proposed as an alternative, where a stable fluidized bed is formed when the adsorbent particles are in equilibrium between the particle sedimentation velocity and the upward liquid flow velocity. EBA, however, is let down by short contact times between the adsorbent and the target analyte, and large particle size, lowering the surface area to volume ratio available for target capture. Other separation technologies, such as filtration represent an attractive alternative for the concentration of product and removal of fouling matter. However, the tiny pore size in these systems introduces a flow resistance, requiring significant pressure to maintain modest flow rates (Yavuz et al. 2009). Furthermore, permeable membranes are susceptible to fouling and as a result, have a relatively short lifespan. In cases where speed of separation is not a critical factor, centrifugation or flocculation may be performed to remove unwanted particulates from the target environment.

Recently, in 2013, the global market for commercial biotechnology separations was estimated at \$17 billion, and is expected to grow at a rate of about 10% for the next five years. Presently, the preferred separation techniques in downstream processing are liquid chromatography and membrane filtration, which together account for 36% of the global biotechnology separations market (Higsmith 2013). An increasingly recognized, alternative technique for the separation of proteins from complex mixtures involves the use of magnetic particles. Advancements in our understanding of the theoretical aspects of magnetism has allowed us to exploit magnetic properties for separation purposes. **This is reflected in the number of articles citing “magnetic separation” over the past ~20 years (Figure 1).** Today, magnetic particles are implemented in a myriad of separation applications in the bioscience

field, from separation of cells and cell organelles, to nucleic acid, antibodies, enzymes, microorganisms and other target analytes (Hawkins 1998; Pankhurst et al. 2003; Safarik and Safarikova 2004; Safarik et al. 1995). **Figure 2** presents the basic principle of magnetic separation, which takes place in three steps.

**\*FIGURE 2 HERE\***

As magnetic particle applications continue to diversify and demonstrate viability, perhaps the area where they offer the most impact, is in downstream processing. Abating the dependence on expensive and laborious affinity chromatography protocols will be pivotal in the reduction of the bottleneck in downstream processing in the future. Magnetic separations offer multiple advantages over conventional chromatography techniques, namely: 1) rapid separation, 2) few handling steps, 3) minimization of protein degradation, 4) reduced system costs, 5) tolerates complex feed streams, 5) gentle process, 6) scalability, and 7) particle compatibility with ligands used in chromatographic systems. Typically, commercially manufactured adsorbents, such as chromatography resins, are largely porous to increase surface area for adsorption. A drawback of this porosity is the ability of the pores to become plugged with foulants. In contrast to porous adsorbents, non-porous magnetic supports are not affected by the presence of particulate matter in the target environment. Additionally, their non-porous nature permits fast binding kinetics due to the elimination of internal diffusion limitations. Furthermore, they can be tailored to possess very large surface areas for increased binding capacities. To compensate for this absence of porosity, magnetic adsorbents are significantly smaller (nanomicon scale) than chromatographic adsorbents, which typically have  $\sim 90\mu\text{m}$  diameter.

Magnetic separation remains largely a lab-scale endeavour. However, the demand for processing of much larger feed stocks for the capture of biologically valuable targets has resulted in progress in this area. Towards this, an integrated process of purifying proteins using low magnetization particles in combination with high gradient magnetic fields has been developed, and is termed high gradient magnetic fishing (HGMF) (Hubbuch et al. 2000). The HGMF process allows adsorption, washing, elution and sanitization of the magnetic adsorbent in a way that permits continuous multi-cycle processing. Although still in its infancy, HGMF pilot plants are in operation and processing volumes of 100L per batch have been described for the purification of antibodies using magnetic adsorbents coated with Protein A (Holschuh and Schwämmle 2005). For a complete list, see (Franzreb et al. 2006). Pilot scale-up efforts such as these are encouraging and represent the potential for magnetic separation in large-

scale industrial environments. However, scaling up a biomagnetic separation process requires well-defined conditions, and is not just a case of using larger magnets and reaction vessels. Instead, the magnetic force, which depends on the magnetization of the magnetic beads, as well as the gradient of the magnetic field over a certain distance, must be clearly defined. If these parameters are not well defined and understood, issues such as particle loss and aggregation are inevitable.

Despite the potential this technology offers, the global market for magnetic separation in 2013 was \$142 million, or < 1% of that for global biotechnology separations. Currently, new separation procedures, including magnetic separations, are projected to show a 13.4% growth over the five year period from 2013-2018 (Higsmith 2013). A number of issues must be addressed if magnetic separation is to become a routine separation technology for biomolecule purification. Primarily the cost of synthesizing the superparamagnetic particles on which the technology is based must be reduced, as well as the scale of production increased. To be competitive with other separation technologies, particles must be tailored to possess properties leading to optimal and reliable performance. Additionally, the particle systems should be robust so that they withstand routine cleaning in place (CIP), and sanitization protocols, permitting particle recycling and reuse.

### *1.1. Comparison of expanded-bed and packed-bed separation*

Packed-bed adsorption, is widely used to isolate selected biological macromolecules from complex mixtures in industrial settings (Harrison et al. 2015; Yavuz et al. 2009). The key issue in the viability of the technique is to design a system in which adsorption and desorption are highly selective and rapid. The performance of an expanded-bed of SPM beads can be compared to packed-bed adsorption using conventional bioseparation theory, based on defined adsorption isotherms, mass balance, and mass transport analysis. In this section, we consider these processes to gain a deeper understanding of the advantages and disadvantages of expanded-bed magnetic bead separation.

Packed-bed adsorption systems dominate industrial downstream processing, and in the simplest case, their performance can be defined in terms of the number of transfer units ( $n$ ) and height of a transfer unit ( $HTU$ ). The value of  $n$  is ideally defined by feed and product properties, while the value of  $HTU$  is determined by the micro and macroscopic chemical and physical properties of the adsorption system. The  $HTU$  of a packed-bed is related to the physical and chemistry properties of the system

$$HTU = G / (K \times a) \quad (1)$$

where  $G$  is the flow rate of the feed stream,  $K$  is the overall mass transport coefficient, and  $a$  is the relative packing area per volume. Consideration of  $HTU$  indicates that for a given set of performance criteria, i.e., feed flow rate, feed concentration and product concentration, the efficiency of the bioseparation process will be improved for higher values of  $K$  and  $a$ .

Expanded-bead separation can, in the simplest case, be considered as a batch process, where the feed is loaded into a stirred tank chemical reactor in which the analyte is in equilibrium with the suspended beads. The operating conditions are defined by a mass balance on the reactor

$$q = q_F + \frac{H}{W}(y_F - y) \quad (2)$$

where  $y$  and  $y_F$  are the initial and final feed concentrations,  $q$  and  $q_F$  is the initial and final concentrations in the adsorbent,  $H$  is the amount of feed, and  $W$  is the amount of adsorbent. In the case in which mass transport is highly efficient, the values of  $q$  and  $y$  are determined by the form of the adsorption isotherms of the analyte on the adsorption matrix. In cases in which mass transport is the rate limiting factor, the resulting system will be less efficient. High binding affinities and mass transfer coefficients will result in high values of  $q$  and low values of  $y$  for a given reaction system. Clearly, higher values of  $W$  and smaller values of  $H$  will also result in a more efficient separation.

Mass transfer conditions play a central role in the performance of adsorption unit operations. In the simplest case, the mass transfer coefficient,  $K$ , for a packed or expanded-bed adsorption system, will be defined by the rate of convective mass transport of the analyte to the beads, i.e., assuming the analyte-bead interaction is not the rate limiting factor. Correlations have been developed to define the mass transport coefficients for these systems due to the important role that separation plays in many industrial processes (Gray and Gray 1966; Schlichting 1960; Sherwood et al. 1975; Treybal 1982). The Mass transfer correlation for a packed-bed,  $k_p$ , has been shown to have the functional form

$$\frac{k_p}{v^3} = 1.17 \left( \frac{d \cdot v^0}{v} \right)^{-0.42} \left( \frac{v}{D} \right)^{-0.67} \quad (3)$$

where  $v^0$  is the superficial velocity,  $d$  is the particle size,  $v$  is the kinematic viscosity of the carrier fluid, and  $D$  is the diffusion coefficient of the analyte. The mass transfer correlation for a dispersion in a stirred suspension,  $k_d$ , is

$$\frac{k_d}{D} = 0.13 \left( \frac{P/V}{\rho \cdot v^3} \right)^{0.25} \left( \frac{v}{D} \right)^{0.33} \quad (4)$$

where  $P/V$  is the power volume and  $\rho$  is the fluid density. Although it is not immediately obvious, these two correlations have similar functional forms, i.e., the mass transfer coefficient scales with  $D$  and  $v$  with a power of 0.66 and -0.20 to -0.25 respectively. The most significant differences between the two adsorption geometries are the particle size for the packed-bed, i.e.,  $d$  of the magnetic beads is typically much smaller than that of a packed-bed, and the mixing conditions of the feed with the adsorbent. Expanded-beds have high mass transfer rates because favorable mixing conditions can be achieved. Packed-beds can also have high mass transfer rates due to their packing densities, but this also leads to high pressure drops.

This analysis allows us to make a number of observations about the use of expanded-beds of magnetic beads for affinity separation of analytes from biomolecular products. As we have seen, expanded and packed-bed separation configurations differ significantly in the number of effective transfer units that can be achieved, which means that expanded-bed separation requires highly specific analyte-bead interactions to achieve reasonable levels of performance. There can also be a significant difference in  $a$ , which can be orders of magnitude smaller if low densities of magnetic beads are used. However, the mass transfer rates of an expanded-bed system can be quite high if the mixing conditions are controlled appropriately. So what are the advantages of the expanded-bed approach? The differentiating feature of expanded-bed separation is its capacity to process large volumes of feedstock rapidly, which has been a constant bottleneck for packed-bed separation techniques. Additionally, pre-filtration processing requirements are minimal, and the ease of cleaning and sterilization protocols is greatly improved using an expanded-bed approach. Clearly, to optimize separation conditions for a dispersion, it is imperative to select a bead surface chemistry that has a significant analyte binding affinity, which is discussed in a subsequent section of this review.

### *1.2. Magnetically activated expanded-bed separation*

Successful implementation of a magnetic expanded-bed separator is highly dependent on the means of isolation of the magnetic beads from the feed stream, and the rinse, elution, and sterilization conditions. Key performance criteria for the separation system are the speed of separation of the magnetic beads from the expanded-bed, the speed of re-suspension, and the

efficiency of recovery of the magnetic separation media, which, we will see, is the key cost driver for this format of adsorption separation.

Laboratory scale magnetic separation is typically performed with a rare-earth permanent magnet or an electromagnet, generating local magnetic fields at their surfaces in the order of 1.5 and 0.5 Tesla, respectively. A key issue in the efficiency of magnetic separation is that the magnetic fields decay exponentially away from the magnet surface with a scaling factor that is defined by the shape of the pole pieces. In the most general case the force,  $F$ , is related to the form of the magnetic field,  $B$ ,

$$F = m \cdot \nabla B \quad (5)$$

where  $m$  is the magnetic moment. In a one-dimensional, high magnitude field, the force  $F$  transduced to a superparamagnetic bead can be simply expressed as

$$F \propto V \cdot M \cdot dB/dz \quad (6)$$

where  $V$  is the volume of the bead,  $M$  is the magnetization of the bead, and  $dB/dz$  is the gradient of the magnetic field at a specific position. The superparamagnetic bead accelerates in the rapidly changing magnetic field to steady state velocities,  $v_o$ , where the magnetic force is equal and opposite to the hydrodynamic drag force

$$F = 6\pi \cdot \mu \cdot R \cdot v_o \quad (7)$$

where  $\mu$  is the viscosity of the fluid phase and  $R$  is the radius of the bead. Practically, this means the separation velocity of a superparamagnetic bead scales with the second power of  $R$  and is directly proportional to the magnitude of  $M$ . Obviously, more efficient separation can be achieved using larger beads that are more magnetic with higher magnetic field gradients.

Large-scale bioseparation is the primary focus of this review, which we define to be of the order of 100 kg. To the best of our knowledge, industrial expanded-bed magnetic adsorption has not been implemented at this scale to date. This is largely due to the difficulty encountered when attempting to effectively remove almost 100% of 1000 liters of magnetic beads from a process stream in under 10 minutes. **Figure 3** presents the results of a finite elements calculation of the efficiency of separation of magnetic beads from the laminar flow of a feed stream through a quadruple magnet as a function of the tube diameter, flow rate, and

$M$  of the magnetic bead. The relative importance of hydrodynamic and magnetic forces have been presented in terms of the Mason number,  $M_a$ ,

$$M_a = \frac{F_{hydro}}{F_{mag}} \quad (8)$$

which have been calculated for a given magnetic separator from (6) and (7). A number of observations can be made about the performance of a magnetic separator from the results presented in **Figure 3**. First, it appears that high levels of recovery of the SPM beads is only achieved when  $M_a < 0.1$ . As the Mason number increases, the number of beads lost in a single stage quadruple magnet increases to approximately 80% for an  $M_a$  of 1. Second, as the magnetization of the beads increases, the Mason number decreases, which makes it possible to work with larger flow rates. Even more significant results can be achieved using larger beads, i.e., the  $F_{mag}$  scales with the second power of the radius of the bead, which suggests that larger beads are better for rapid processing of magnetic expanded-bed. Third, similar levels of separation efficiency can be achieved for larger diameter separators and higher flow rates if the Mason number is held constant.

\*FIGURE 3 HERE\*

A number of practical issues need to be raised at this point in our discussion of the design of large-scale magnetic separators. First, although larger magnetic beads are easier to separate, smaller beads have a higher surface area to volume ratio, i.e., the area per unit volume ( $a$ ) scales as the inverse of  $R$ , and consequently, are desirable from a handling and cost perspective. Second, the simulations presented in **Figure 3** do not take into account the fact that the magnetic beads recovered from the process fluid have finite volume and that the recovery of large volumes of beads result in a significant decrease in the flow cross-section of the separator as they accumulate. Third, the recovery of beads from the separator needs to be performed in a manner in which almost all the magnetic beads are recovered, meaning that the smallest beads will dictate the overall speed of separation, due to them moving slowest and therefore, represent the rate-limiting step. For larger scale separations, high gradient magnetic separation (HGMS) has been used to separate weakly magnetic, or very small magnetic nanoparticles, from suspension, while maintaining high particle binding capacities (Hubbuck and Thomas 2002a; Moeser et al. 2004a). HGMS units have been described that consist of a magnetisable steel wool mesh, through which the magnetic nanoparticle suspension passes, resulting in retention of the nanoparticles by magnetization of the steel wool matrix. Magnetic

fields generated in the steel wool are typically achieved using an electromagnet, with a flux density up to 1.3 Tesla, resulting in capture of up to 90% of the nanoparticles in the size range of 10 nm (Moeser et al. 2004b).

The analysis in the last two sections highlights the advantage of expanded-bed magnetic separation in downstream processing, i.e., speed and simplification of the overall downstream system requirements. In our opinion, industrial scale magnetic bioseparation is currently limited by the availability of appropriate magnetic adsorption materials to achieve rapid and high levels of recovery of analyte, at a cost comparable to conventional packed-bed adsorption materials.

## *2. Superparamagnetic (SPM) beads and their properties*

Table 1 presents a list of commercially available superparamagnetic beads and their physical properties, targeted at laboratory scale separation. Magnetic microparticles in the size range of 1 to 2  $\mu\text{m}$  are typically used in these applications, due to the relatively low quantities of analyte that need to be purified, and the relatively low magnetic field gradients needed for their separation in volumes up to  $\sim 50$  ml. The magnetic mobility of the particles is largely dependent on their loading of inorganic magnetic material, which is presented in the final column of Table 1. Magnetic polystyrene particles have been synthesized with 55% wt. magnetite (Zheng et al. 2005) and polymer-magnetite composite particles have been synthesized with 30 – 40% wt. magnetite (Omi et al. 2001) Dynabeads contain between approximately 17% and 32% wt. maghemite depending on the size of the microparticle (Fonnum et al. 2005). Higher magnetic loadings are desirable when a large number of measurements need to be performed rapidly. In addition to speed of separation, low magnetic loadings result in particle loss during the functionalization, extraction and washing steps. Size distributions and uniformity of magnetic loading must be considered, as variation in these parameters can lead to loss of valuable analyte during separation.

For biopharmaceutical separations, SPM beads should ideally exhibit high surface area and magnetic mobility, permitting rapid separations, while using minimum quantities of SPM beads. Microparticle uniformity is critical, as the presence of small micro or nanoparticles in suspension leads to longer separation times, and can potentially lead to the loss of smaller microparticles over time, and consequently, the associated loss of product bound to the beads. Additionally, magnetic particles should have a coefficient of variation (CV) less than 15% for large scale applications to ensure the particles come out of solution at similar magnetic velocities, when an external magnetic field is applied. Due to the inverse relationship

between the surface area per unit volume ( $A/V$ ) and the radius of the particle, higher surface areas are typically achieved by using smaller magnetic nanoparticles. However, there is a trade-off, as smaller magnetic particles display lower magnetic mobility, which scales with the square of the particle radius (**Figure 4**). Fortunately, in dense suspensions of magnetic particles, dipole-dipole interactions result in the formation of chains of magnetic particles that align in the direction of the field gradient. These chains have a lower effective  $M_a$ . This means that highly efficient magnetic separation of 0.3  $\mu\text{m}$  superparamagnetic beads, that are 90% by weight magnetite, can be performed using permanent magnets, at bead densities of approximately  $10^8$  beads/mL.

\*FIGURE 4 HERE\*

In summary, although ubiquitously used for laboratory-scale bioseparations, magnetic particles have yet to be fully embraced for larger industrial scale purification of biomolecules. This is mainly attributed to the high synthesis cost of commercial magnetic microparticles and lack of suitable surface chemistries. It has previously been estimated that for magnetic separation to become commercially viable for large scale purification, the cost of magnetic particles needs to be reduced  $\sim 1000$  fold, assuming the binding capacity of the beads is 100 mg/g (Flickinger 2013). There is therefore a need to develop more economic methods for large-scale synthesis of microparticles with uniform sizes and high magnetic mobility.

### *2.1 Superparamagnetic nanoparticle synthesis*

Superparamagnetism is a state of matter where the direction of the magnetic moment of the single domain nanoparticle can freely move between two stable orientations on the easy axis due to thermal energy. In the absence of an external magnetic field, the magnetization of the nanoparticles averages zero, due to flipping of the orientation of the magnetic field in the nanoparticle (**Figure 5**).

\*FIGURE 5 HERE\*

Particles exhibiting superparamagnetism should not interact with each other in the absence of a strong magnetic field, which would lead to the formation of unwanted aggregates. Importantly, this property allows re-dispersion of the magnetic particles after removal of a magnetic field. Ferromagnetic and ferrimagnetic materials consist of magnetic domains,

regions in which the direction of magnetization is mostly uniform. If a ferromagnetic particle is reduced below a threshold size, it will consist of a single magnetic domain. The threshold size for magnetite ( $\text{Fe}_3\text{O}_4$ ) is about 40 to 50 nm at room temperature. Below this size, magnetite consists of a single magnetic domain, and the orientation of the magnetic moment within this domain can be in one of two directions along the easy axis.

Commonly cited SPM materials include iron, nickel, cobalt, gadolinium oxide, and iron oxide. Magnetite ( $\text{Fe}_3\text{O}_4$ ) and maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ) are the most widely used superparamagnetic nanoparticles due to their low toxicity, well-established synthesis routes, and high saturation magnetization. The most common synthesis method for iron oxide nanoparticles is the co-precipitation of ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) ions in a basic medium, using salts such as iron (III) chloride or iron (II) sulphate (Massart 1981; Sugimoto and Matijević 1980). Precipitation occurs by the formation of nuclei, which grow uniformly by diffusion of Fe ions from the solution to the surface of the forming crystal (Mahmoudi et al. 2011). The crystal growth must be controlled to produce nanoparticles below the superparamagnetic size threshold of 20 nm (Sun and Zeng 2002). Also common is the synthesis of iron oxide nanoparticles by the microemulsion method, where an iron precursor, such as ammonium iron (II) sulphate, precipitates as iron oxide inside aqueous droplets or reversed micelles, suspended in an organic continuous phase (Inouye et al. 1982) This method in theory offers greater size control than co-precipitation, as the nanoparticle size is governed by the droplet or micelle size. Alternative bio-synthesis routes for the synthesis of iron oxide nanoparticles include magnetotactic bacteria and genetically engineered ferritin protein cages (Lang et al. 2007; Uchida et al. 2006). However, these techniques have yet to be fully realized for large-scale production of nanoparticles.

## *2.2 Synthesis of microparticles*

Iron oxide nanoparticles of the size of 10 nm are suitable for many applications such as magnetic resonance imaging (MRI) contrast agents, drug delivery, cell separation, and water treatment. (Franzreb et al. 2006; Gupta and Gupta 2005; Oberteuffer et al. 1975). However, for purification applications, due to the smaller size of nanoparticles, stronger magnetic field gradients (approximately  $10^4$  T/m) are required to manipulate the nanoparticles allowing efficient separation from suspension at reasonable time scales. The incorporation of large amounts of iron oxide nanoparticles into micron and sub-micron-sized structures produces an additive effect of the magnetic moments of the individual nanoparticles, while still retaining the superparamagnetic properties of the nanoparticles. Due to the combined magnetic

moments, micron-sized particles are significantly more responsive to magnetic field gradients than the individual nanoparticles, allowing them to be separated from suspension with cheaper, less powerful permanent magnets (approximately 100 T/m) (Borlido et al. 2013). It is therefore more commonplace in laboratory settings to use 100 nm to 1000 nm assemblies of superparamagnetic nanoparticles for detection and purification of analytes.

**Figure 6** presents three strategies for the assembly of superparamagnetic iron oxide nanoparticles into microparticles. Nanoparticles have been described that are distributed in a polymer microparticle matrix (Levison et al. 1998; Liu et al. 2003; Ugelstad et al. 1983; Yang et al. 2008). Alternatively, nanoparticle self-assembly to form a tightly packed spherical SPM has also been reported. In this case, the microparticle is subsequently coated with a polymer layer to provide chemical groups to which biomolecules such as DNA and proteins can be appended (Muzard et al. 2012; Uhlen 1989). This type of polymer-coated magnetic microparticle is commonly referred to as a core-shell structure. Thirdly, microparticles have been synthesized by precipitating maghemite nanoparticles inside the pores of polymer microparticles previously formed via emulsion polymerization (Häfeli 1997; Ugelstad et al. 1983). A fourth category, where superparamagnetic nanoparticles are physically or chemically adsorbed onto a micron sized polymer particle has also been reported. However, this technique results in microparticles with very low magnetic loading due to the polymer core constituting the majority of the particle volume (Bizdoaca et al. 2002). The synthesis of polymer magnetite composites is typically accomplished through suspension, or mini emulsion-templated polymerization, where nanoparticles or nanoparticle clusters are suspended in monomer containing droplets which are polymerized through the addition of a polymerization initiator, encapsulating the nanoparticles. (Csetneki et al. 2004; Hai et al. 2009; Ma et al. 2005; Ramírez and Landfester 2003) While the polymer component of these particles is a useful support for coupling functional groups for different applications, the magnetic loadings are generally low, in the order of 30 to 40% wt (Hai et al. 2009; Ramírez and Landfester 2003; Zheng et al. 2005).

\*FIGURE 6 HERE\*

Particles with higher magnetic loadings have recently been synthesized using emulsion-templated synthesis of magnetic particles, where the dispersed phase consists solely of iron oxide nanoparticles. Emulsification techniques include membrane emulsification, (Shang et al. 2006; Yanagishita et al. 2009) shear devices, (Goubault et al. 2001; Mabelle et al. 1999;

Mason and Bibette 1997) and microfluidic droplet devices (Martín-Banderas et al. 2006; Sugiura et al. 2001; Umbanhowar et al. 1999). Each technique has advantages and disadvantages in terms of throughput, ease of fabrication, use, expense and robustness.

### *2.3 Particle post-synthesis coating and functionalization*

Following synthesis, naked magnetic beads must be coated to prevent degradation or aggregation of the particles. Furthermore, metals such as iron are sensitive to oxidation. To reduce these unwanted outcomes and increase chemical stability, the integrity of the SPM bead is preserved by coating it with an outer layer. Typically, organic polymers and surfactants, or inorganic layers, such as silica or alumina are chosen. The aim is to enhance either, or both, of the repulsive forces – steric repulsion and electrostatic interaction, thereby reducing the propensity of the beads for aggregation. In addition to increasing stability, often these layers confer chemical moieties to the particle for further chemical modification or direct conjugation to various biomolecules/ligands (Berry 2009). Due to its ease of use and different functional groups available in the form of commercial silanes, silica represents the most popular choice for inorganic coating. However, other examples such as alumina, zirconium manganese ferrites, gold and silver have also been reported for the generation of inorganic magnetic sorbents. Organic polymer coatings can be divided into natural polymer coatings, including biocompatible polysaccharides such as dextran, starch, heparin, pullulan, chitosan and alginate. Non-natural polymer coatings including polyvinylpyrrolidone (PVP)(Guowei et al. 2007), provide increased steric repulsion between particles due to their long hydrophobic chains. Other examples of non-natural polymer coatings include polyvinyl alcohol (PVA)(Liu et al. 2008), polyethylimine (PEI), polymethylmethacrylate (PMMA)(Gass et al. 2006), poly-2-methacryloyloxyethyl phosphorylcholine (PMPC) and polyamidoamine (PAMAM) (Strable et al. 2001).

The ability to tightly control the polymer thickness and grafting density is highly desirable for tailored design of functional magnetic adsorbents. This can be achieved using surface-initiated polymerisation techniques, such as atom transfer radical polymerisation (ATRP) chemistry. Bioconjugation of a variety of biological entities, such as biotin (Kang et al. 2009), antibody fragments (Iwata et al. 2008) and peptides (Glinel et al. 2008) have been made possible using the ATRP approach. After synthesis and coating, the resulting particle has the appearance of a superparamagnetic core and an outer layer consisting of a polymer, which often provides specific functional groups for bioconjugation to affinity ligands (see **Figure 7**).

For conjugation of protein-based ligands to the particle surface, there are four functional group options available – primary amines (NH<sub>2</sub>), carboxyls (COOH), sulfhydryls (SH) and carbonyls (CHO). Commonly, peptides and proteins are immobilized through free amine groups using EDC/NHS crosslinking chemistry (Bartczak and Kanaras 2011). Coupling to carbonyl groups is commonly performed using hydrazine crosslinkers (Moghimi and Moghimi 2008). Thiol-reactive groups for coupling to Cys-containing affinity ligands include maleimides, iodoacetamides and disulfides, and may be conjugated to magnetic matrices via the use of sulfo-SMCC crosslinking agents (Kalia and Raines 2010), as depicted in **Figure 7**. The versatility of bi-functional crosslinkers makes them extremely useful for enabling covalent immobilization between two previously incompatible functional groups, and should be considered in the design of affinity ligand-functionalised biomaterials.

Also noteworthy is the use of Biotin-avidin technology, which is regularly exploited in magnetic separation. Specific nucleic acids are routinely recovered from crude samples by immobilizing biotinylated, complimentary strands on streptavidin-coated magnetic particles (Mojsin et al. 2006). While it is generally accepted that orientating the ligand in a site-specific manner is beneficial in affinity-ligand adsorbent design, the role of other variables, such as ligand density and spacer arm length are less understood. Spacer arms become necessary when the non-binding domain of the ligand does not offer substantial spacing between the structure of the adsorbent and the ligand binding domain to permit the ligand to interact freely with the target. The significance of spacer arm length has been previously reported in the literature (Fuentes et al. 2006) (Hubbuch and Thomas 2002b). Computational analysis and simulations on the effect of linker length also testify that longer spacer arms decrease negative steric effects (Ghaghada et al. 2005; Jeppesen et al. 2001).

### *3. Synthetic affinity ligands for bioseparation applications*

Until the advent of affinity chromatography, highly selective and efficient protein purification was troublesome and challenging. Initially, affinity chromatography protocols were restricted to the use of naturally occurring substrates and inhibitors against the target, immobilized on chromatographic supports. Subsequently, genetic engineering advancements made possible the tagging of expressed proteins with well characterized affinity tags, such as glutathione and histidine tags. Affinity purification of these tagged proteins is easily achieved using (glutathione-S-transferase) GST-modified matrices, or in the case of histidine, by IMAC (immobilized metal affinity chromatography), using nickel or cobalt functionalized supports (Hochuli et al. 1988; Lichty et al. 2005). Synthetic triazine dyes, such as Cibacon Blue FG-

3A, have also been widely used for their selective ability to bind most proteins, and their resistance to chemical and biological degradation (Dean and Watson 1979). However, these synthetic dyes are let down by their observed leaching into the eluate, leading to concerns over their application in the purification of biologics destined for human administration. Another class of synthetic affinity ligands demonstrating promise are DNA-based aptamers. Their observed high stability and specificity makes them attractive synthetic alternatives for ligands in protein downstream processing. However, screening these oligonucleotides via SELEX (systematic evolution of ligands by exponential enrichment) remains expensive and time-consuming (Walter et al. 2012). More recently, synthetic peptides have been proposed as affinity ligand alternatives. In recent years, the emergence and evolution of peptide phage display technology, alongside developments in solid phase peptide synthesis (SPPS), molecular modelling and *in silico* design, has rapidly accelerated the recognition of peptides as synthetic affinity alternatives, with improved performance characteristics for bioseparation applications. Another class of synthetic ligands garnering increasing interest, are miniaturized antibody fragments, which can be expressed from synthetic DNA constructs, and exhibit several beneficial features over full-length antibodies for the separation of biomaterial (Figure 8).

\*FIGURE 8 HERE\*

In 2015, the biopharmaceutical market is expected to reach \$239 billion (BCCResearch 2011). Coinciding with this, will be increasing demands for therapeutic proteins, while maintaining increasingly stringent regulatory protocols. This pressure for demand will require industry to embrace new technology for efficient and cost-effective downstream protein purification. A significant reduction in downstream cost can be achieved by adoption of synthetic affinity ligands over traditional monoclonal antibodies, for bioseparation assays. In the following section, we review and discuss two important types of synthetic ligands, in the form of peptides and miniaturized antibody fragments, as economical affinity ligand alternatives in the downstream processing of valuable proteins.

### 3.1 Synthetic peptides

In 2012, peptide-based drugs were the second most approved drug by the FDA, after small molecules (Osborne 2013). Already, there are over 100 peptide-based drugs on the market, with the top sellers generating billion-dollar sales annually (Craik et al. 2013). Despite this, relatively little attention has been focused on peptide application in industry as affinity ligands for the purification of therapeutically valuable proteins. There is a wealth of published evidence suggesting that small, low molecular weight, synthetic, peptide-based ligands may offer an effective alternative to protein-based ligands for bioseparation applications. This is due to a combination of attractive properties peptides possess, such as high chemical and biochemical stability, low immunogenicity, affinity and specificity similar to conventional protein-based ligands, and a synthetic nature - permitting flexibility for optimizing interaction with the target. Developments in solid-phase peptide synthesis (SPPS), originally described by Merrifield (Merrifield 1963) now allows routine, inexpensive synthesis of peptides by biotech companies in large quantities (Loffet 2002). SPPS also makes possible the inclusion of chemical moieties that act to increase peptide stability or tailor the binding characteristics for a particular application, making it a very powerful tool. Examples of this include the incorporation of non-naturally occurring amino acid side chains and/or inclusion of D-stereoisomers, to increase ligand stability by increased resistance to degradation by peptidases. The synthetic approach to affinity ligand design imparts less of a contamination risk, as the ligands are not derived from animal sources. As a result, regulatory concerns over contamination and infectious agents in the final product are eliminated. Issues over leaching of immunogenic antibodies are also circumvented. Moreover, manufacturing costs for synthetic peptides (\$50-75/g of peptide) are dramatically lowered compared to the production of biologics (recombinant Protein A can cost up to \$17,000/L of resin) (Chen 2009). Additionally, as a result of the high affinity (~ nM) antibodies possess for their targets, often harsh elution conditions are required. This is unfavorable as it can lead to chemical degradation of the resin with prolonged use. Furthermore, such low pH elution favors antibody aggregates in the product which must be removed, thereby lowering yield and increasing costs. In general, peptides display weaker affinities for their targets, permitting the use of milder elution conditions, extending the column lifetime, and preventing the formation of aggregates. In agreement with this, a hexamer peptide targeting human fibrinogen has been identified (Kaufman et al. 2002). The authors describe a low ionic strength buffer at pH 4 to elute fibrinogen from the column with high purity and almost no loss of activity. **Importantly, peptides also exhibit exceptional selectivity, and have been successfully used to sequester and**

purify proteins from crude starting sources such as soy whey mixtures, whole blood and milk (Fields et al 2012, Noppe et al 2006).

Given these desirable attributes, it is unsurprising that a diversity of peptides have been identified in an effort to replace expensive biologics, while still maintaining chemical stability, as well as high specificity and affinity for their targets. Towards this, a tripeptide tetramer ((Arg-Thr-Tyr)<sub>4</sub>-Lys<sub>2</sub>-Lys-Gly) has been described that binds to the Fc portion of IgG (Fassina et al. 2001) and yields approximately 95% pure protein. It also demonstrates a broad range of specificities for antibodies from a variety of hosts (i.e. horse, pig, human, cow, rat, mouse, rabbit, goat, sheep and chicken) as well as multiple IgG classes (Fassina et al. 1998). This is an improvement on Protein A, which does not bind goat and chicken antibodies. This synthetic tripeptide tetramer has been further modified to replace all amino acids with D stereoisomers, which generated a protease-resistant variant, able to maintain its IgG binding properties after multiple sanitization procedures (Verdoliva et al. 2002). This provides a nice demonstration of the power and flexibility of the synthetic route, where ligand performance was improved by careful manipulation of the peptide sequence. Other reports of synthetic peptides capable of binding antibodies with increased binding capacities and purity levels >93% have also been described (Lund et al. 2012). Replacing the expensive capture step in mAb downstream processing by using peptides instead of protein-A-based supports, will undoubtedly act to alleviate unremitting economic concerns in therapeutic mAb purification. Table 2 presents a list of synthetic peptides that have been successfully implemented in affinity purification procedures.

### *3.2 Multimeric peptide variants*

Small, linear peptides represent an exciting class of compounds for bioseparation as demonstrated in the examples mentioned previously. However, a new generation of peptide ligands, termed “multimeric”, or “branched” peptides are garnering more interest than their linear counterparts, on behalf of the benefits afforded by multimerization. These include higher affinities due to avidity effects and resistance to enzymatic proteolysis - two desirable properties when designing ligands for bioseparation or therapeutic application.

Generally, small peptide-ligands will not exhibit very high affinities with their targets (~ $\mu$ M), which can be explained by the flexibility and conformational freedom observed in short peptide molecules. Furthermore, short-chain peptides will have a limited number of residues available to make contact with the target (Terskikh et al. 1997). The ability to withstand enzymatic proteolysis and digestion is a common goal when designing any peptide-based

ligand and is an imperative property for reusability and recycling. Multimeric peptide structures are understood to be more resistant to protease attack as a result of the observed steric interference formed when multivalent peptides are generated. This observation has previously been reported (Falciani et al. 2007). The use of multivalent peptides as affinity reagents has also been documented. One study describes the synthesis of a multimeric peptide targeting TNF alpha. In this case, the authors noted an enhanced binding of the peptide upon multimerization (Fassina et al. 1992a). Similarly, in another study by the same group, the authors reported affinities of at least two orders of magnitude higher for the multivalent peptides, compared to their monomeric counterparts (Fassina et al. 1992b). Notably, synthetic multimeric peptides have also been used for the affinity purification of antibodies (Butz et al. 1994). Other examples include a multivalent peptide species targeting streptavidin with a reported increase in binding affinity of two orders of magnitude over a monovalent counterpart (Bastings et al. 2011) . Similarly, Helms et al describe the generation of a multivalent peptide targeting collagen and a subsequent increase in binding affinity of >2 orders of magnitude compared to monovalent formats (Helms et al. 2009). Recently, our lab has described the capture of herpes simplex virus and anticancer proteins from soy whey, using multimeric peptide-coated magnetic particles (Fields et al. 2012; Ran et al. 2014). Thus, it is clear that the properties afforded by multiple simultaneous interactions may differ substantially compared to monovalent interactions, and that the benefits afforded by these branched molecules can be exploited in the next generation of affinity reagents.

### *3.3 Miniaturized antibody fragments*

The seminal work of Kohler and Milstein in the 1980s, allowed the continuous production of antibodies of pre-determined specificity by fusion of antibody-producing B-cells with an immortal cell line (Köhler and Milstein 1975). While antibodies remain the gold standard for affinity-based reagents, their production is not without limitations. Owing to the requirements of post translational modifications, such as glycosylation of the C<sub>H2</sub> domain, full-length antibodies must be expressed in mammalian cell culture. Mammalian cell culture facilities cost several hundred million dollars, require years to establish, and the cell culturing is typically slow, expensive and plagued with issues such as cellular toxicity and apoptosis (Dietmair et al. 2012). Due to advancements in antibody engineering in recent years, full-length antibodies can now be dissected, and expressed as minimal binding fragments, lacking the glycosylated constant domains. Consequently, fully functional antibody fragments may be inexpensively expressed in bacteria. While the Fc portion of antibodies is important for

mediating humoral immune responses, this functionality is largely unnecessary for affinity purification purposes. Moreover, the lack of an Fc domain in antibody fragments is advantageous due to the greatly reduced non-specific binding to this region. Reduction in non-specific binding using antibody fragments in place of full length IgGs has previously been reported for immunohistochemistry protocols (Brandon 1985). Importantly, engineering full-length antibodies to express only the minimal binding fragment retains the parental affinity for the target, as the paratope is unaltered.

### *3.4 Synthetic Antibody Fragments*

Recently, a comprehensive protocol detailing the design, assembly and prokaryotic production of synthetic genes encoding antibody fragments, has been reported by our group. It describes identification of genes encoding the V<sub>H</sub> and V<sub>L</sub> domains of antibodies, followed by custom gene synthesis and direct cloning into an appropriate vector, for recombinant expression (Fields et al. 2013). This approach permits the synthesis of genes, and even entire genomes, “*de novo*”, without the need for pre-existing DNA sequences (Itakura et al. 1977; Re et al. 2007). As a result, completely synthetic, double-stranded DNA oligomers are made available.

Synthetic approaches to antibody fragment design, means they are amenable to sequence variation, to modulate and improve their binding characteristics. For example, IgGs may be humanised for therapeutic application, by genetic techniques, but additionally, other properties such as affinity, half-life and probing function can all be tweaked and optimized (Deng et al. 1994; Dubreuil et al. 2005; Eisenhardt et al. 2007). This ability to alter the binding profile of fragments gives researchers tremendous power. With the recent emergence of new DNA technology and custom gene synthesis, rapid, affordable and accurate synthesis of DNA is now widespread. In the last decade alone, the price of custom gene synthesis has dropped from \$10/base pair, to just \$0.35/base pair (Genscript Inc). This cost is expected to decrease in the future, as automation becomes more commonplace. Importantly, the synthetic route to DNA production guarantees sequence accuracy and absence of mutation in the final DNA construct. Moreover, sequences can be manipulated and optimized to enhance recombinant expression in *E.coli* strains, by techniques such as codon optimization, which has been shown to enhance yields of expressed scFv fragments 14-fold (Sharma et al. 2014). Furthermore, sequences can be modified to alter binding parameters like specificity and

affinity. These processes are largely facilitated by the recent prevalence of web-based applications, as well as molecular and structural databanks. As a result, this contemporary approach to antibody fragment design is largely a human-guided, computer-assisted endeavor.

### *3.5 Antibody fragment applications*

Antibodies and their fragment counterparts share similar application potential to peptides, in that they are essential tools for basic research, laboratory diagnosis and in medicine. As miniaturized antibody fragments retain the parental specificity and affinity of monoclonal antibodies, it is perhaps unsurprising that these smaller counterparts have been successfully used for affinity purification procedures. The ability to use humanized scFv fragments offers advantages over animal-derived monoclonal antibodies, which can leak into the product, resulting in an immunogenic response in the patient (Burton 1996). Furthermore, scFv fragments can be rapidly expressed in bacterial cells, to high titers, at an inexpensive cost compared to the production of monoclonal antibodies via hybridoma technology. Importantly, non-specific binding to contaminants is reduced since the size of the molecule has been refined to the minimal binding fragment. For a list of antibody fragments acting as affinity ligands, see Table 3 below.

### *4. Concluding remarks and outlook*

The drive towards smaller, synthetic molecules with high affinity and specificity provides a much needed development in the field of protein downstream processing. Examples reported to date have demonstrated the promise synthetic ligands offer as affordable, scalable and reliable alternatives to monoclonal antibody-based affinity separation. The flexibility and facile nature of the synthetic route makes it a powerful option, especially when paired with bioinformatics and/or computational design to optimize ligand-target interactions. In an ongoing effort to abate the dependence on chromatography in downstream processing, alternative affinity matrices, in the form of magnetic particles, have emerged as a viable candidate. As reviewed here, these supports have demonstrated success in a myriad of bioseparation assays, and tolerate large processing volumes, alluding to their industrial scale potential. Although not yet fully embraced by industry leaders, magnetic separation, in combination with adoption of synthetic affinity ligands, offers a complimentary approach to help alleviate speed, production and economic concerns in the downstream purification of valuable proteins.

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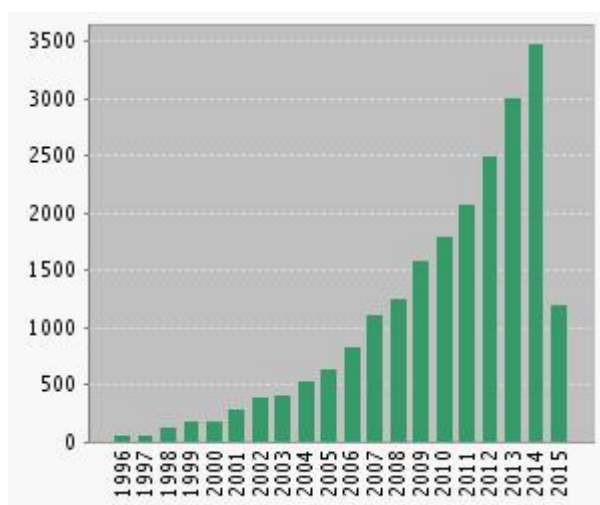
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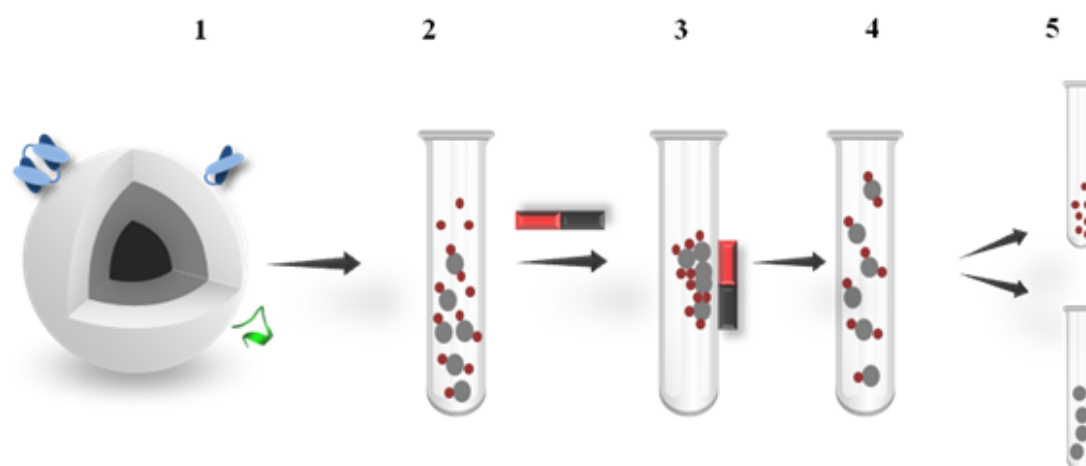
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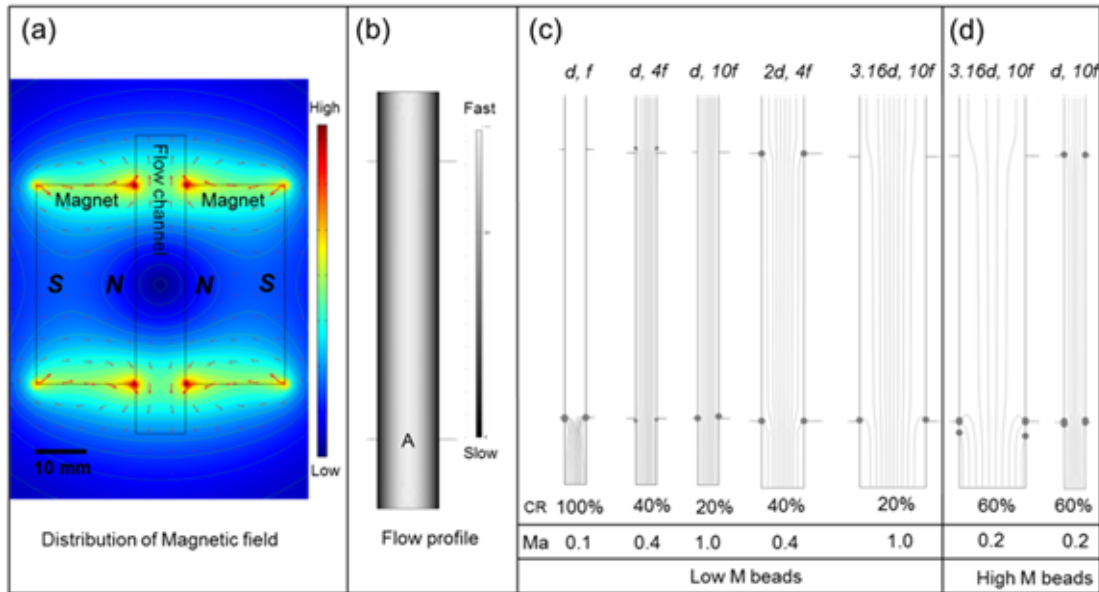
## Figure Captions



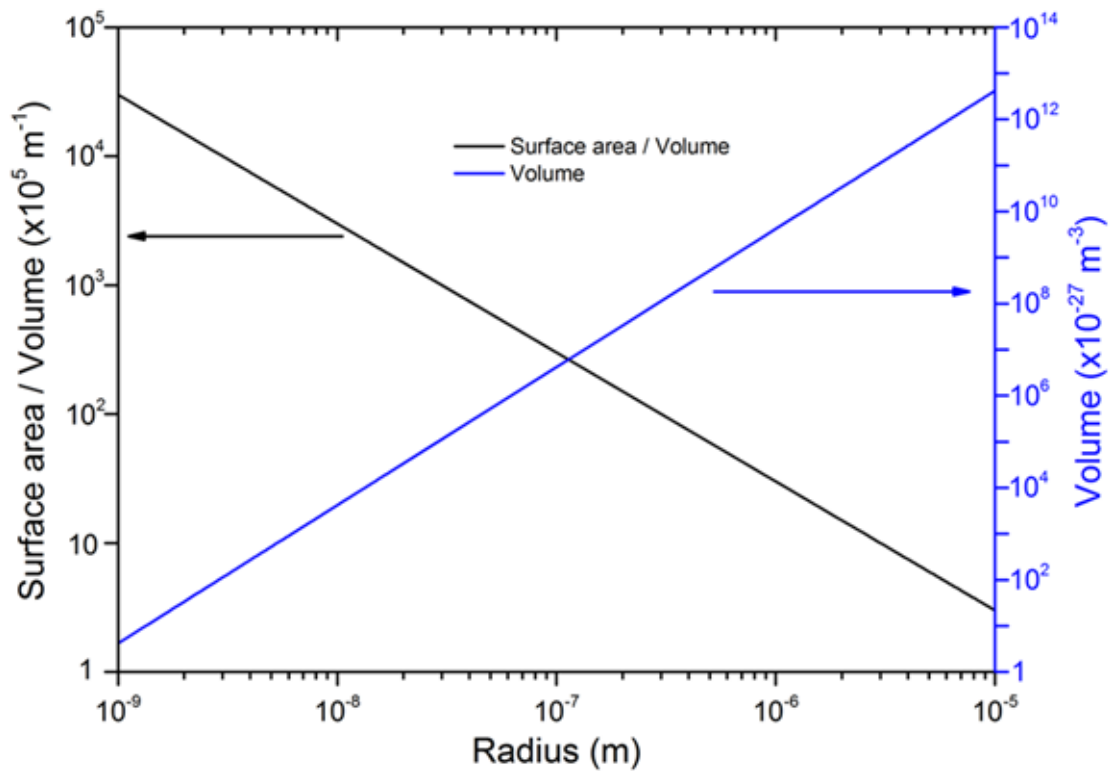
**Figure 1. Number of articles citing “magnetic separation” over the past ~20 years.** Numbers were obtained from the Thomson Reuters’ Web of Knowledge search (May 2015).



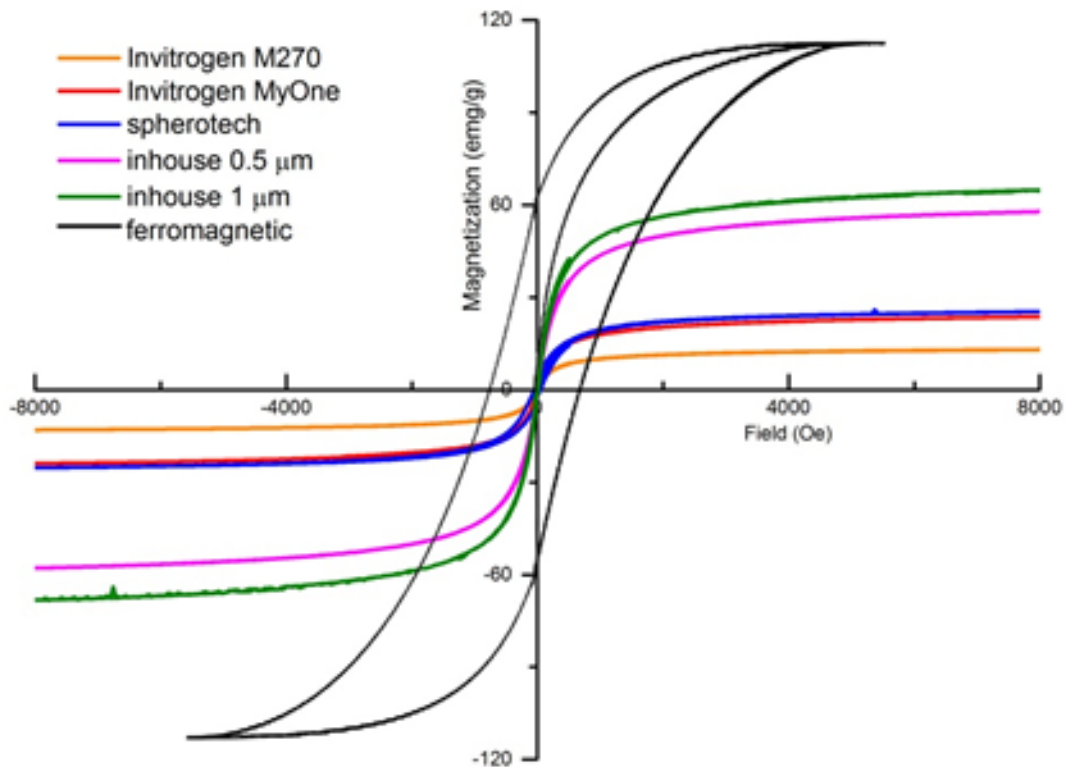
**Figure 2. Overview of the magnetic separation procedure.** 1) Magnetic particles (grey) are appropriately functionalised with an affinity ligand (antibody, antibody fragment (blue), peptide (green)) and incubated alongside the sample containing the target analyte (red) 2). Magnetic beads bound to the target are collected by application of an external magnet 3), before the beads are washed to remove any non-specifically bound material 4). Finally, the target protein is eluted from the magnetic particles using suitable elution buffers 5).



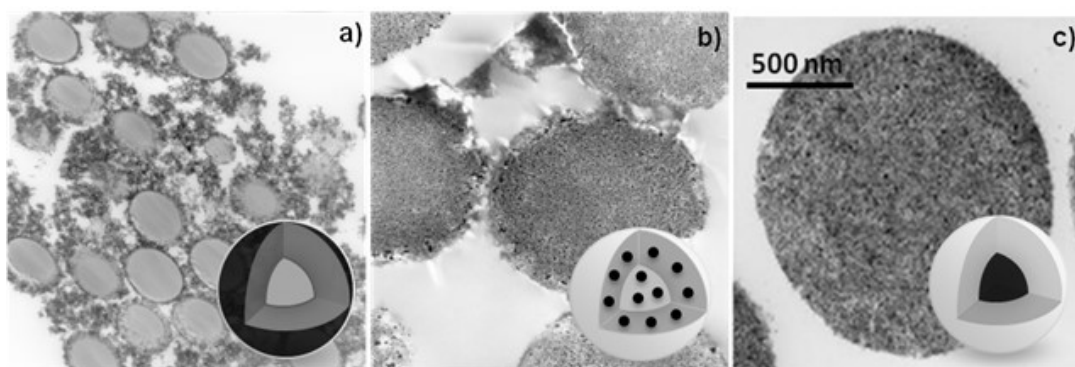
**Figure 3. Magnetophoretic separation of SPM beads in a quadrupole magnet extractor.** **a)** Finite element simulation of the magnetic field produced by a NdFeB quadrupole assembly for separation of SPM beads flowing through a channel. The SPM beads are introduced under flow conditions from the bottom inlet and captured on the sidewall of the channel. **b)** Finite element modeling flow profile in the channel. Laminar flow in the channel shows a parabolic flow profile with fast flow velocity in the center of the channel and low flow velocity near the channel wall **c-d)** SPM bead capturing trajectories at different flow rates and channel widths. **c)** Capturing rates (CR, defined as the percentage of captured beads of the total number of beads introduced in the channel) for 1  $\mu\text{m}$  SPM beads, with a density of  $1.7 \times 10^3 \text{kg/m}^3$  and saturation magnetization of  $20 \text{ am}^2/\text{kg}$ , in channels with different flow rates. In this study, the channel diameters were  $d$ ,  $2d$ , and  $10d$  (where  $d=3.16 \text{ mm}$ ) and the flow rates were  $f$ ,  $4f$ , and  $10f$  (where  $f=2.3 \text{ ml/min}$ ). The CR decreased from 100% to 20% as the flow rate increased from  $f$  to  $10f$ . **d)** Using SPM beads with higher saturation magnetization increased the CR three times. These SPM beads have a density of  $2.6 \times 10^3 \text{kg/m}^3$  and a saturation magnetization of  $100 \text{ am}^2/\text{kg}$ . The separation efficiency of SPM beads in flow conditions is defined by the Mason number,  $Ma$ , which is the ratio of the drag force to the magnetophoretic force on a bead. Figures (c-d) present Mason numbers of the bead at position “A” in the channel (Figure 2b) for different flow configurations.



**Figure 4. Scaling laws for nano- and micrometer beads.** This figure depicts the relationship between the volume (blue) and the radius of the bead, as well as the ratio of surface area to volume ( $A/v$ ) and the bead radius (black). Data was calculated for spherical beads with a radius in the range of 1 nanometer to 10 microns. The volume of the bead decreases rapidly as the radius of the bead decreases. This in turn results in a large increase in the surface area to volume ratio.

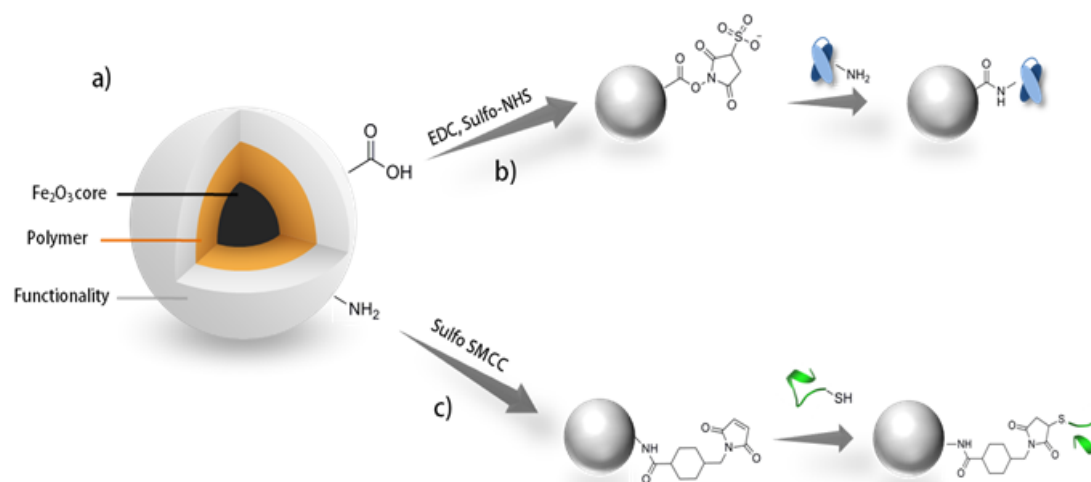


**Figure 5. Magnetic properties of superparamagnetic beads and ferromagnetic materials.** This figure presents squid (superconducting quantum interference device) magnetometric curves for five types of SPM beads and a ferromagnetic material (cobalt) at room temperature. In contrast to the ferromagnetic materials, there is no noticeable hysteresis loop for the SPM materials. The high nanoparticle content of our in-house SPM beads (Shang et al. 2006) results in significantly higher saturation magnetization compared to commercial beads.

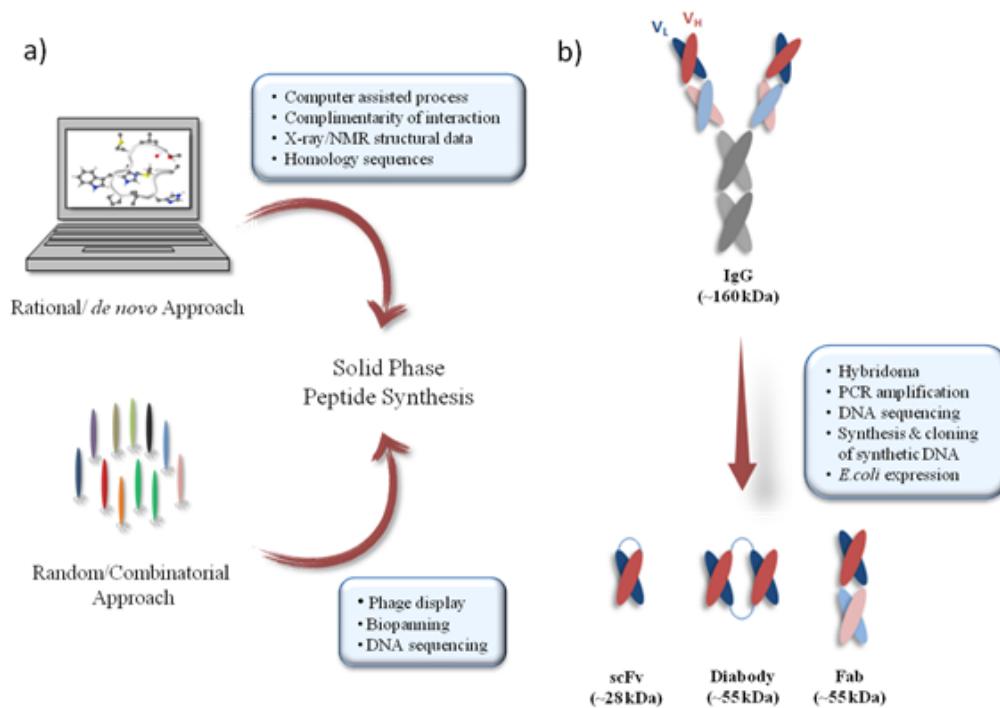


**Figure 6. Schematic and TEM images of commonly used magnetic bead constructs.** **a)** Depicts a core-shell particle structure where a dense magnetic shell is coated on a polymer core. The magnetic content in these particles ranges from 10%-20% wt. TEM image depicts a cross-section through beads embedded in a wax matrix. **b)** Depicts a distributed particle structure in which the nanoparticles have precipitated within a polymer matrix. Magnetic loadings in this case, can be up to 32% wt. **c)**

Represents a solid core particle structure where a dense magnetic core is coated with an ultra-thick polymer layer. Magnetic loading in core-shell particles is typically >70% wt.



**Figure 7. Schematic of multi-layered bead-ligand bioconjugation with common amine and carboxyl functionalities.** **a)** Image depicting the layered particle structure, showing the  $\text{Fe}_2\text{O}_3$  superparamagnetic core, the surrounding polymer shell, and the resulting chemical functionality conferred to the particle. **b)** Schematic of conjugation of antibody fragments to a carboxyl-derivatized particle surface, using EDC and sulfo NHS chemical crosslinkers (Muzard et al. 2012). **c)** Schematic of conjugation of a thiolated synthetic peptide to an amine-derivatized bead using sulfo-SMCC crosslinking chemistry (Fields et al. 2012). In each case, the immobilized ligand is irreversibly and covalently tethered to the magnetic support.



**Figure 8. Routes to generation of synthetic affinity ligands. a)** Rational and random approaches for the generation of peptide-based affinity ligands. In cases where prior knowledge of target protein structures is available, a *de novo*, or rational approach to ligand design may be possible. This scenario involves computational methods to deduce desired structural features necessary for binding to target proteins. Frequently, this information is limited and structural data for certain proteins may not be available. In this case, a random, or combinatorial approach may be adopted, where libraries of bacteriophage expressing ( $10^9$ ) random peptide sequences may be screened to identify peptides with affinity for a given protein. **b)** Synthetic miniaturized antibody fragments are readily prepared by first obtaining a hybridoma cell line secreting the antibody of interest. Next, the genetic material is sequenced after performing PCR to amplify the relevant segments of DNA. The DNA is subsequently synthesized. A full protocol has recently been described (Fields et al. 2013). The emergence of new DNA technologies and inexpensive custom gene synthesis procedures permits synthesis of accurate and flexible sequences encoding scFv, diabody and/or Fab fragments, which retain affinity and specificity of the parental IgG.

Peptide Sequence	Target	Support	Reference
((Arg-Thr-Tyr) <sub>4</sub> -Lys <sub>2</sub> -Lys-Gly	IgG	Sepharose	(Fassina et al. 2001)
Asp-Ala-Ala-Gly	IgG	Agarose	(Lund et al. 2012)
His-Trp-Arg-Gly-Trp-Val	IgG	Toyopearl amino-650M resin	(Menegatti et al. 2012)
His-Trp-Arg-Gly-Trp-Val *	Human IgG	Toyopearl amino-650M resin	(Yang et al. 2009)
(Thr-Pro-Arg) <sub>14</sub> *	Trypsin	Agarose	(Makriyannis and Clonis 1997)
(D-His-Pro-Phe-His-Leu-Leu-Val-Tyr)	Renin	Sepharose	(McINTYRE et al. 1983)
Glu-Tyr-Lys-Ser-Trp-Glu-Glu-Phe *	Human blood coagulation factor VIII	Monolith	(Amatschek et al. 2000)
Phe-Leu-Leu-Val-Pro-Leu	Fibrinogen	Toyopearl amino-650M resin	(Kaufman et al. 2002)
(Gly-Ala-Met-His-Leu-Pro-Trp-His-Met-Gly-Thr-Leu) <sub>4</sub>	Bowman-Birk Inhibitor	Magnetic particle	(Fields et al. 2012)
Val-D-Leu-Pro-Phe-Phe-Val-D-Leu	Pepsin	Magnetic particle	(Filuszová et al. 2009)
Val-D-Leu-Pro-Tyr-Phe-Val-D-Leu *	Gastric aspartic proteases	Magnetic Particle	(Rajčanová et al. 2012)
Arg-Val-Arg-Ser-Phe-Tyr *	Von Willebrand factor	Toyopearl resin	(Huang et al. 1996)
Trp-His-Trp-Arg-Lys-Arg	$\alpha$ -lactalbumin		(Gurgel et al. 2001)
His-Trp-Trp-Trp-Pro-Ala-Ser	Insulin	Sepharose	(Yu et al. 2004)
Asp-Gln-Asp-Gln-Asp-Thr *	Lactoferrin	Monolithic gel	(Noppe et al. 2006)
Ser-Trp-Trp-Arg-His-Ala-Ala-Val-Tyr-Glu-Trp-Asp *	$\alpha$ -cobratoxin	Agarose	(Byeon and Weisblum 2004)
Tyr-Tyr-Trp-Leu-His-His	Staphylococcal enterotoxin B	Toyopearl amino-650M resin	(Wang and Carbonell 2005)

**Table 2.** Selected examples of synthetic peptide-based affinity separations.

\*Denotes where more than one peptide is described

<b>Antibody Fragment</b>	<b>Target</b>	<b>Support</b>	<b>Ref</b>
scFv	Reteplase	Sepharose 4B	(Guo et al. 2006)
scFv	Tumor Necrosis Factor $\alpha$	Sepharose 4B	(Abdolalizadeh et al. 2013)
scFv	IFN-alpha2b	Cellulose	(Hil'chuk et al. 2005)
scFv/diabody	Bowman-Birk inhibitor	Magnetic particle	(Muzard et al. 2012)
Single domain (VHH)	Ice Structuring protein (ISP)	Sepharose 4B	(Verheesen et al. 2003)
Fv fragment	Cytochrome c oxidase	Sepharose	(Kleymann et al. 1995)
Fv fragment	Ubiquinol cytochrome c oxidoreductase	Sepharose	(Kleymann et al. 1995)
scFv	Hepatitis B virus surface antigen	Sepharose 4B	(Canaan-Haden et al. 1995)

**Table 3.** Selected examples of antibody fragment-based affinity separations.

<b>Manufacturer</b>	<b>Product</b>	<b>Diameter (µm)</b>	<b>Surface Functionalities</b>	<b>Composition</b>	<b>Density (g/cm<sup>3</sup>)</b>	<b>Magnetism</b>	<b>Magnetic Content</b>
<b><i>Invitrogen</i></b>	Dynabeads (MyOne, M270, M-450)	1, 2.8, 4.4	Carboxylic acid, Streptavidin, antibodies, protein, antigens, DNA/RNA	Iron oxide nano particle core with polystyrene shell	1.4-1.7	Superparamagnetic	37%
<b><i>Chemicell, GmbH</i></b>	SiMAG	0.5, 0.75, 1	Silanol, ion-exchange groups, hydrophobic group, amine, protein, antibodies, antigens, DNA	Maghemite nano particles in non or highly porous silica matrix	2.25	Superparamagnetic	n.a.
<b><i>Spherotech Inc.</i></b>	SPHREO magnetic particles	1-120	Amino, Carboxyl, Diethylamino, Dimethylamino, Hydroxyethyl	Magnetite nano particles coated on a polystyrene core	n.a.	Superparamagnetic or paramagnetic	~15%
<b><i>Emd Millipore</i></b>	Pure Proteome magnetic beads	0.3, 1, 2.5, 10	Carboxyl, Streptavidin, Protein, N-hydroxy-succinimide (NHS)	Magnetic nano particles in a silica, or polystyrene matrix	n.a.	Paramagnetic	n.a.
<b><i>Thermo Scientific</i></b>	Pierce magnetic beads	1, 1-10	Streptavidin; Protein; NHS; Antibodies, glutathione	Magnetite nano particles coating/or in polymer particle	2	Superparamagnetic	n.a.
<b><i>GE Lifescience</i></b>	Sera-mag magnetic beads	1	Carboxyl, Streptavidin, Neutraavidin, Oligo, Amine, Protein	Magnetite nano particles and polystyrene core	1.5, 2.0	Superparamagnetic	~40%, ~60%
<b><i>Polysciences Inc.</i></b>	Biomag	~1.5	Carboxyl, Streptavidin, Amine, Oligo (dT), Antigen, Antibody	Silanized iron oxide matrix, irregular shape	2.5	Superparamagnetic	n.a.

**Table 1.** Comparison of commercially available magnetic constructs. n.a refers to data that was not available at the time of writing